

## EXPERIMENTAL STUDY

**Effect of Gubenfangxiao decoction on respiratory syncytial virus-induced asthma and expression of asthma susceptibility gene orosomucoid 1-like protein 3 in mice**

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**Huang Zhengguang, Gao Lijuan, Zhao Xia, Ling Hao, Chen Wenjun**, Institute of Pediatrics of Nanjing University of Chinese Medicine, Jiangsu Key Laboratory of Pediatric Respiratory Disease, Nanjing 210023, China**Supported by** Natural Science Foundation of China (Effect of Gubenfangxiao Decoction on the Expression of Asthma Susceptibility Gene ORMDL3 and ADAM33 and its Signaling pathway, No. 81173209)**Correspondence to: Prof. Zhao Xia**, Institute of Pediatrics of Nanjing University of Chinese Medicine, Jiangsu Key Laboratory of Pediatric Respiratory Disease, Nanjing 210023 China. [zhaoxiahy@126.com](mailto:zhaoxiahy@126.com)**Telephone:** +86-25-86798182**Accepted:** May 16, 2015**Abstract****OBJECTIVE:** To investigate the effect of Gubenfangxiao decoction (GBFXD) on respiratory-syncytial-virus (RSV) -induced asthma and the expression of asthma susceptibility gene, orosomucoid 1-like protein 3 (ORMDL3) in mice.**METHODS:** Seventy-two female BALB/c mice were randomly assigned to normal, model, GBFXD high dose, GBFXD moderate dose, GBFXD low dose and montelukast groups. An asthma model was induced via intraperitoneal injection and aerosol inhalation of ovalbumin (OVA) and repeated intranasal instillation of RSV in all mice, except those in the normal group. All treatments were administered at the first onset of asthma (within 8 weeks of model establishment) and the mice were euthanized after 28 days of treatment. The levels of transforming growth factor- $\beta$  (TGF- $\beta$ ) and interleukin-6 (IL-6) in bronchoalveolar lavage fluid (BALF) of the mice

were measured and the expression of asthma susceptibility gene ORMDL3 in lung tissue was determined using real-time polymerase chain reaction (RT-PCR) and western blotting.

**RESULTS:** Expression of ORMDL3 and levels of TGF- $\beta$  and IL-6 were significantly higher in the model group ( $P < 0.05$ ,  $P < 0.01$ ) compared with the normal mice. Levels of ORMDL3, TGF- $\beta$  and IL-6 were significantly lower in all three GBFXD treated groups ( $P < 0.05$ ) compared with the model group. However, the levels in the GBFXD treatment groups did not differ significantly from the montelukast group.**CONCLUSION:** GBFXD had a therapeutic effect in this experimental model. The functional mechanism of GBFXD may involve multiple factors, including alleviation of airway inflammation, down-regulation of asthma susceptibility gene ORMDL3 and inhibition of airway remodeling.© 2016 JTCM. Open access under [CC BY-NC-ND license](https://creativecommons.org/licenses/by-nc-nd/4.0/).**Key words:** Asthma; ORMDL3 protein, mouse; Airway remodeling; Gubenfangxiao decoction**INTRODUCTION**Bronchial asthma is chronic inflammation of the airway, with both genetic and environmental factors implicated in its pathogenesis.<sup>1,2</sup> Recurrent asthma attacks involve infiltration of inflammatory cells and abnormal changes to the structure of the airway. The mechanism underlying airway remodeling has yet to be determined. Recently, the first asthma-specific, ge-

nome-wide association study (GWAS) identified orosomucoid 1-like protein 3 (ORMDL3) as a candidate asthma risk gene,<sup>3</sup> in multiple ethnic groups.<sup>4,6</sup> However, the function of ORMDL3 remains unknown and further studies are needed to elucidate the mechanisms of ORMDL3 and its association with asthma. Gubenfangxiao decoction (GBFXD) is prepared using a formula containing eleven Chinese herbs. The formula was designed by Professor Jiang Yuren for the management of asthma and has been used in Jiangsu Province Hospital of Traditional Chinese Medicine (TCM) for more than 30 years. Our previous studies showed that GBFXD could protect against airway inflammation and inhibit airway hyperresponsiveness.<sup>7</sup>

The aim of the present study was to investigate the effect of GBFXD on respiratory-syncytial-virus (RSV) -induced asthma and the expression of the asthma susceptibility gene ORMDL3, in mice.

## METHODS

### Drug and reagents

GBFXD was prepared from a TCM formula consisting of Huangqi (*Radix Astragali Mongolici*), Dangshen (*Radix Codonopsis*), Baizhu (*Rhizoma Atractylodis Macrocephalae*), Fuling (*Poria*), calcining Muli (*Concha Ostreae*), Chantui (*Periostracum Cryptotympanae*), Chenpi (*Pericarpium Citri Reticulatae*), Fangfeng (*Radix Saposhnikoviae*), Xinyi (*Flos Magnoliae Biondii Immaturus*), Wuweizi (*Fructus Schisandrae Chinensis*) and stir-frying with liquid adjuvant Gancan (*Radix Glycyrrhizae*). The ratio of mass in grams used in the formula was 5:3.3:3.3:3.3:5:2:2:1:2:2:1 and the granules were supplied by Jiangsu Province Hospital of Traditional Chinese Medicine. The decoction was produced using the granules and double distilled water at a concentration of 2.25 g/mL. Montelukast sodium tablets (20070058, Hangzhou MSD pharmaceutical Co., Ltd., Hangzhou, China) were used to prepare a solution of approximately 0.16 mg/mL, in sterile distilled and stored at 4 °C. Ovalbumin (OVA) was supplied by Sigma (St. Louis, MO, USA). Respiratory syncytial virus (RSV) was obtained from the Institute of Pediatrics of Nanjing University of Chinese Medicine (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-13 and IL-6 were supplied by eBioscience (San Diego, CA, USA). Real-time PCR master mix Plus (SYBR Green) and RNA extraction kits were supplied by Takara Biotechnology (Shanghai, China). Monoclonal antibodies against ORMDL3 were supplied by Abcam (Massachusetts, MA, USA) and against  $\beta$ -actin by Santa Cruz (California, CA, USA). Secondary antibodies were also supplied by Abcam. Primers for IL-13, ORMDL3 and  $\beta$ -actin were designed and synthesized by Takara Biotechnology.

### Animals and asthma model establishment

Seventy-two healthy, female, 6-week-old BALB/c mice

[certificate of quality No. SCXK (Hu) 2012-0002], weighing  $[(18 \pm 2) \text{ g}]$  were supplied by the Laboratory Animal Center of Shanghai Slack Co., Ltd., (Shanghai, China). Mice received food and water ad libitum for 1 week before experiments [temperature at  $(24 \pm 1) ^\circ\text{C}$  and humidity at  $50\% \pm 5\%$ ]. Mice were maintained in the Laboratory Animal Center in the Nanjing University of Traditional Chinese Medicine. All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nanjing University of Traditional Chinese Medicine.

Mice were randomly divided into six groups: normal, model, GBFXD high dose, GBFXD moderate dose, GBFXD low dose and montelukast. Asthma was induced in all mice, except those in the normal group. A sensitization and challenge protocol was employed, as described previously,<sup>8,9</sup> with small modifications. Mice were immunized *via* intraperitoneal injection of 100  $\mu\text{g}$  OVA, conjugated to 1 mg aluminum potassium sulfate, in a total volume of 0.2 mL on the 1st and 8th day. Mice inhaled aeroallergens as 2.5% OVA, every day from the 15th to the 28th day, for 30 min. Asthma was induced using intranasal instillation of 50  $\mu\text{L}$  RSV on the 29th, 42nd, and 55th day. From the 29th to the 55th day, mice were challenged with inhalation of 2.5% OVA solution for 30 min, once every 3 days.<sup>10</sup>

### GBFXD treatment

The standard dose of GBFXD was based on individual animal body weight. The formula used was  $\text{Db} = \text{Da} \times \text{Rab}$ , which was derived from the formula used to determine the standard dose for humans.<sup>11</sup> The formula used for humans was based on a ratio of 24 g crude medicine to 1 kg of murine body weight. GBFXD high-dose (GBFXD-H) was 36 g/kg, the moderate dose (GBFXD-M) was 24 g/kg and the low dose (GBFXD-L) was 12 g/kg. GBFXD and montelukast acetate (2.6 mg/kg) treatments were intragastrically administered, once a day from the 2nd day of model establishment. The normal and model groups were given an equal volume of distilled water, intragastrically at the same time points. All mice were euthanized after 28 days of treatment.

### RSV

RSV supernatants were prepared according to the method described previously.<sup>12</sup> The 50% tissue culture infective dose (TCID<sub>50</sub>) of RSV was measured as  $1.0 \times 10^{6.5} \text{ TCID}_{50}/\text{mL}$ .

### Bronchoalveolar lavage fluid collection

Mice were sacrificed 24 h after the final treatment. BALF was acquired as described previously.<sup>7</sup> Three aliquots of 0.5 mL PBS were injected and withdrawn through a tracheal cannula. Following centrifugation, the supernatant was stored at  $-70 ^\circ\text{C}$ , for measurement of IL-6 and TGF- $\beta$  concentrations, using ELISA.

### ***Lung histology and morphometry***

The left lungs of mice were fixed in 4% buffered paraformaldehyde for at least 24 h. Transverse sections of 5  $\mu$ m were harvested. Each section was stained with hematoxylin and eosin for histological assessment using light microscopy. Additional sections were stained with Masson's trichrome to detect collagen deposition and periodic acid Schiff (PAS) stain to show mucin within goblet cells. For sections stained with Masson's trichrome, the region of interest was a 20  $\mu$ m band, immediately beneath the epithelium, as this is considered to include the ECM and contractile elements associated with the airways.<sup>13</sup> The positive peribronchiole areas stained with Masson's trichrome were quantified using light microscopy connected to an Image-Pro Plus image analysis system (Media Cybernetics, Chicago, IL, USA). The numbers of PAS-positive epithelial cells (goblet cells) in individual bronchioles were counted as previously described.<sup>14</sup> At least six bronchioles were counted on each slide and all the histological analyses were performed blind to treatment.

### ***RT-PCR determination of ORMDL3 expression in lungs***

The lungs of mice were collected after the final treatment. Total RNA from lung tissue was extracted using TRIzol reagent, according to the manufacturer's instructions. The concentration of RNA was measured using spectrophotometry. Total RNA was reverse transcribed to cDNA, using reverse transcriptase reagents from Takara, according to the manufacturer's protocol. Real-time polymerase chain reaction (RT-PCR) was carried out using SYBR Green II fluorescent dye method, on Applied Biosystems 7500 PCR apparatus (California, CA, USA). The sequences of primers used are shown in Table 1.

### ***Western blotting***

Protein was extracted from lungs using protein lysate from Beyotime Biotechnology (Haimen, China). It contained 50 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100  $\mu$ g/mL phenylmethylsulfonyl (PMSF) and 1  $\mu$ g/mL aprotinin. The protein concentration was measured using a BCA protein assay kit from Pierce (Rockford, IL, USA). Sample aliquots, containing 30  $\mu$ g protein, were fractionated on 12% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes, from Millipore

(Massachusetts, MA, USA). The membranes were blocked with 5% non-fat milk in 1  $\times$  Tris-buffered saline, with 0.1% Tween-20 (TBST) for 2 h at room temperature and incubated overnight with anti-ORMDL3 (1 : 500 final dilution) antibody. After incubation, the blots were washed with 1  $\times$  TBST (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 0.1% Tween-20) 3 times for 10 min. Membranes were then incubated with secondary antibody (1 : 5000 final dilution) at room temperature for 2 h and washed 3 times, for 5 min, with 1  $\times$  TBST. The blots were visualized using a chemiluminescence system from Pierce, according to the manufacturer's instructions. The results were normalized using  $\beta$ -actin densitometry on an Image-Pro Plus image analysis system.

### ***Statistical analysis***

All data were analyzed by using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). Differences between groups were evaluated with one-way analysis of variance.  $P < 0.05$  was considered significant.

## **RESULTS**

### ***Histopathological examination***

Compared with the normal group, there was little eosinophil infiltration and thicker airway and alveolar walls in the model group. The levels of eosinophil infiltration in the GBFXD treatment groups were lower than in the model group ( $P < 0.01$ ). However, there were no significant differences among the groups treated with GBFXD and montelukast ( $P < 0.05$ , Figure 1). Compared with the normal group, collagen deposition throughout the lung interstitium, around the wall of airway, was significantly different ( $P < 0.01$ ) in the model group. There was a significant reduction in collagen deposition in the GBFXD and montelukast treatment groups ( $P < 0.05$ , Table 2 and Figure 2). The number of goblet cells that stained positively for mucin was significantly increased in the model group compared with the normal group. The GBFXD treatment groups showed a sharp decrease in the presence of mucin compared with the model group ( $P < 0.05$ , Table 3 and Figure 3).

### ***Levels of IL-6 and TGF- $\beta$ in BALF supernatant***

The IL-6 and TGF- $\beta$  levels were very low in the nor-

Table 1 The sequences of forward and reverse primers of ORMDL3

Gene	Primer	Product annealing
GAPDH	Forward: 5'-cgtgttctctaccccaatgt-3'	104 bp 58 $^{\circ}$ C
	Reverse: 5'-tgtcactacttggcaggtttct-3'	
ORMDL3	Forward: 5'-cactgggagcagatggactac-3'	106 bp 58 $^{\circ}$ C
	Reverse: 5'-tggacttggctactacttgggt-3'	



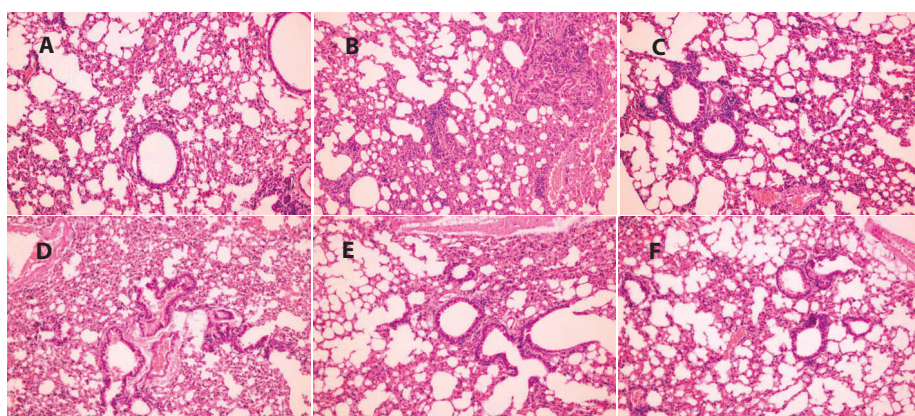


Figure 1 Histopathological analysis of a lung section (HE staining,  $\times 100$ )

A: airway inflammation of normal group; B: airway inflammation of model group; C: airway inflammation of montelukast group; D: airway inflammation of GBFXD-H group; E: airway inflammation of GBFXD-M group; F: airway inflammation of GBFXD-L group. The normal group was treated with distilled water only; the model group was exposed to OVA and treated with distilled water; the montelukast group was exposed to OVA and treated with montelukast acetate (2.6 mg/kg); the GBFXD-H group was exposed to OVA and treated with GBFXD (36 g/kg); the GBFXD-M group was exposed to OVA and treated with GBFXD (24 g/kg); the GBFXD-L group was exposed to OVA and treated with GBFXD (12 g/kg). OVA: ovalbumin; GBFXD: Gubenfangxiao decoction; GBFXD-H: Gubenfangxiao high-dose decoction; GBFXD-M: Gubenfangxiao moderate dose decoction; GBFXD-L: Gubenfangxiao low dose decoction. HE: hematoxylin-eosin. Normally, few eosinophils could be seen in the lung, while OVA sensitization and challenge led to significantly augmented recruitment of eosinophils to the airway (B vs A,  $P < 0.01$ ). Both montelukast and GBFXD treatment effectively diminished tissue infiltration of eosinophils (C, D, E, F vs B,  $P < 0.05$ ).

mal group and significantly elevated in the model group. IL-6 and TGF- $\beta$  levels in the DBFXD groups were significantly lower than those in the model group ( $P < 0.05$ ). There were no significant differences between the levels among the GBFXD and montelukast groups. The results of the enzyme-linked immunosorbent assay indicated that GBFXD ameliorated airway inflammation in mice (Table 3).

#### **Changes in ORMDL3 mRNA expression in lung tissue**

The relative quantity of ORMDL3 mRNA expression in lung tissue of the model group ( $2.30 \pm 0.19$  fold change) was significantly higher than that of the normal group ( $P < 0.05$ ,  $P < 0.01$ ). Compared with the model group, ORMDL3 mRNA expression in all treatment groups was downregulated ( $P < 0.05$ ,  $P < 0.01$ ). There were no significant differences in expression among the GBFXD and montelukast groups ( $P > 0.05$ , Table 3 and Figure 4).

## **DISCUSSION**

In this study, GBFXD reduced the levels of Th2-type cytokines, IL-6 and TGF- $\beta$ , in an RSV enhanced asthma model. Investigations have shown that IL-6 can promote allergen-induced epithelial cell damage, goblet cell hyperplasia with mucus hyperproduction and the production of TGF- $\beta$ .<sup>21</sup> TGF- $\beta$  has also been reported to play a detrimental role in promoting airway remodeling during chronic inflammation associated with asthma. Therefore, GBFXD showed a positive effect by inhibiting Th2-type cytokines and airway remodeling.

Subepithelial fibrosis represents an important feature of asthmatic bronchi. In our study, even at 4 weeks af-

ter the final allergen exposure the model group mice developed universal collagen deposition, which may contribute to a prolonged airway inflammatory response. GBFXD could significantly reduce collagen deposition. We also examined goblet cells, which are the main source of mucin glycoproteins. Interestingly, GBFXD not only inhibited mucin production and collagen deposition but also reduced IL-6 and TGF- $\beta$  levels. This suggests that the GBFXD effects on mucin production and collagen deposition may be associated with the suppression of Th2 cytokines.

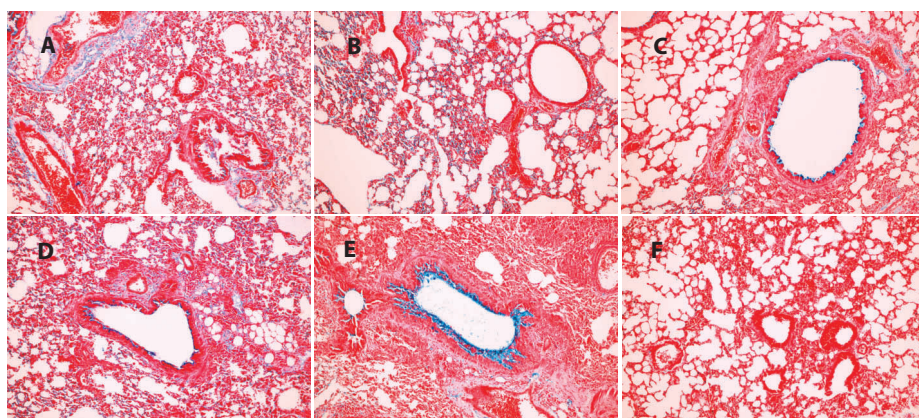
ORMDL3 has been identified as a candidate risk gene for asthma. Consistent with this finding, ORMDL3 was significantly increased in the blood of recurrent wheeze patients.<sup>22</sup> ORMDL3 proteins are negative regulators of sphingolipid synthesis, which may contribute to the development of childhood asthma.<sup>23</sup> ORMDL3 was recently shown to be involved in  $\text{Ca}^{2+}$  signaling and the unfolded protein response (UPR), which can lead to inflammatory responses in asthma.<sup>24</sup> In our study, GBFXD had a significant effect on ORMDL3 expression, at both mRNA and protein levels. ORMDL3 is an inducible lung epithelial gene that regulates oligoadenylate synthetase (OAS) genes,<sup>25</sup> which suggests it may play an antiviral role in RSV.<sup>26</sup>

In conclusion, this study provides evidence that by reducing airway inflammation, decreasing airway collagen deposition, inhibiting airway mucus production and downregulating ORMDL3 expression, GBFXD treatment can prevent the development of chronic asthma. However, the mechanism of action of GBFXD on ORMDL3 expression and its function in the pathogenesis of asthma remains unclear. It is possible that ORMDL3 selectively regulates activation of the endoplasmic reticulum localized transcription factor, ATF6 $\alpha$ , which is linked to the sarcoendoplasmic

Table 2 Changes in collagen deposition for each group ( $\bar{x} \pm s$ )

Group	<i>n</i>	Collagen staining area ( $\mu\text{m}^2/\mu\text{m} \%$ )	PAS positive epithelial cells (%)	ORMDL3/actin
Normal	12	8.11 $\pm$ 1.20	6.78 $\pm$ 0.34	0.15 $\pm$ 0.07
Model	12	43.35 $\pm$ 5.52 <sup>a</sup>	25.99 $\pm$ 1.18 <sup>a</sup>	2.27 $\pm$ 0.05 <sup>a</sup>
Montelukast	12	26.02 $\pm$ 2.13 <sup>b</sup>	13.46 $\pm$ 1.17 <sup>b</sup>	0.10 $\pm$ 0.03 <sup>b</sup>
GBFXD-H	12	19.05 $\pm$ 1.66 <sup>b</sup>	12.54 $\pm$ 1.88 <sup>b</sup>	0.07 $\pm$ 0.03 <sup>b</sup>
GBFXD-M	12	14.05 $\pm$ 1.36 <sup>b</sup>	13.24 $\pm$ 0.33 <sup>b</sup>	0.12 $\pm$ 0.04 <sup>b</sup>
GBFXD-L	12	17.05 $\pm$ 1.34 <sup>b</sup>	14.68 $\pm$ 0.58	0.48 $\pm$ 0.10 <sup>b</sup>

Notes: the normal group was treated with distilled water only; the model group was exposed to OVA and treated with distilled water; the montelukast group was exposed to OVA and treated with montelukast acetate (2.6 mg/kg); the GBFXD-H group was exposed to OVA and treated with GBFXD (36 g/kg); the GBFXD-M group was exposed to OVA and treated with GBFXD (24 g/kg); the GBFXD-L group was exposed to OVA and treated with GBFXD (12 g/kg). OVA: ovalbumin; GBFXD-H: Gubenfangxiao decoction high dose; GBFXD-M: Gubenfangxiao decoction moderate dose; GBFXD-L: Gubenfangxiao decoction low dose; PAS: periodic acid Schiff; ORMDL3: orosomucoid 1-like protein 3. Compared with the normal group, <sup>a</sup> $P < 0.01$ ; compared with the model group, <sup>b</sup> $P < 0.01$ .

Figure 2 Staining as assessed using morphometry for collagen deposition (Masson's staining,  $\times 100$ )

A: collagen deposition of normal group; B: collagen deposition of model group; C: collagen deposition of montelukast group; D: collagen deposition of GBFXD-H group; E: collagen deposition of GBFXD-M group; F: collagen deposition of GBFXD-L group. The normal group was treated with distilled water only; the model group was exposed to OVA and treated with distilled water; the montelukast group was exposed to OVA and treated with montelukast acetate (2.6 mg/kg); the GBFXD-H group was exposed to OVA and treated with GBFXD (36 g/kg); the GBFXD-M group was exposed to OVA and treated with GBFXD (24 g/kg); the GBFXD-L group was exposed to OVA and treated with GBFXD (12 g/kg). OVA: ovalbumin; GBFXD: Gubenfangxiao decoction; GBFXD-H: Gubenfangxiao high-dose decoction; GBFXD-M: Gubenfangxiao moderate dose decoction; GBFXD-L: Gubenfangxiao low dose decoction. Mucus overproduction was evident in the model group (H vs G,  $P < 0.01$ ). Mucus production was clearly reduced in the lungs of GBFXD and montelukast treatment groups (C, D, E, F vs B,  $P < 0.05$ ).

Table 3 The levels of IL-6 and TGF- $\beta$  in BALF supernatant for each group ( $\bar{x} \pm s$ )

Group	<i>n</i>	Dose (g/kg)	IL-6 (pg/mL)	TGF- $\beta$ (ng/mL)
Normal	12	-	74.070 $\pm$ 7.842	3.145 $\pm$ 0.021
Model	12	-	281.101 $\pm$ 18.522 <sup>a</sup>	3.402 $\pm$ 0.081 <sup>a</sup>
Montelukast	12	0.0026	150.478 $\pm$ 4.272 <sup>b</sup>	3.068 $\pm$ 0.03 <sup>b</sup>
GBFXD-H	12	36	64.478 $\pm$ 9.217 <sup>b</sup>	3.235 $\pm$ 0.026 <sup>b</sup>
GBFXD-M	12	24	42.745 $\pm$ 12.276 <sup>b</sup>	3.221 $\pm$ 0.058 <sup>b</sup>
GBFXD-L	12	12	51.762 $\pm$ 12.841 <sup>b</sup>	3.147 $\pm$ 0.060 <sup>b</sup>

Notes: the normal group was treated with distilled water only; the model group was exposed to OVA and treated with distilled water; the montelukast group was exposed to OVA and treated with montelukast acetate (2.6 mg/kg); the GBFXD-H group was exposed to OVA and treated with GBFXD (36 g/kg); the GBFXD-M group was exposed to OVA and treated with GBFXD (24 g/kg); the GBFXD-L group was exposed to OVA and treated with GBFXD (12 g/kg). OVA: ovalbumin; GBFXD-H: Gubenfangxiao decoction high dose; GBFXD-M: Gubenfangxiao decoction moderate dose; GBFXD-L: Gubenfangxiao decoction low dose; BALF: bronchoalveolar lavage fluid; TGF- $\beta$ : transforming growth factor- $\beta$ ; IL-6: interleukin-6. Compared with the normal group, <sup>a</sup> $P < 0.01$ ; compared with the model group, <sup>b</sup> $P < 0.01$ .

reticulum,  $\text{Ca}^{2+}$  ATPase (SERCA2b), and implicated in airway remodeling in asthma.<sup>27</sup> Further investiga-

tion of GBFXD is required to elucidate its mechanism of action.

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